EFFECTS OF DRIED PLUM POLYPHENOL EXTRACT ON BONE FORMATION

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To my dearest parents, beloved husband, and sisters
Without their love, support,
encouragement, and most
of all their patience it would
have been impossible
to accomplish.

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Credit for the research work that has led to the preparation of this dissertation goes to many special individuals. It is the result of this effort that shall not be gone unrecognized. This thesis may be considered the final signature to this work, but it is an open door to further discoveries in the field of science. I greatly appreciate the support of my family, advisor, committee members, friends and colleagues who have made this possible.

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LIST OF ABBREVIATIONS

BMD	Bone Mineral Density
BMC	Bone Mineral Content
ALP	Alkaline Phosphatase
COL	Collagen Type I
OC	Osteocalcin
ON	Osteonectin
OP	Osteopontin
TRAP	Tartrate Resistant Acid Phosphatase
RANK	Receptor Activator of Nuclear Factor-kappaB
RANKL	Receptor Activator of Nuclear Factor-kappaB Ligand
OPG	Osteoprotegerin
Ca	Calcium
PTH	Parathyroid Hormone
IL-1	Interleukin-1
IL-6	Interleukin-6
TNF-α	Tumor Necrosis Factor-α
PGE_2	Prostaglandin E_2
Runx2	Runt-Related Gene 2
IGF-I	Insulin Like Growth Factor-I

TGF-β	Transforming Growth Factor- β
BMP	Bone Morphogenetic Protein
FDA	Food and Drug Administration

CHAPTER I

INTRODUCTION

1.1 Background

Osteoporosis is a chronic silent disease and is considered a major public health threat in the United States [25]. Currently, about ten million individuals suffer from this debilitating disease and approximately 34 million people have low bone mass, which increases their risk of osteoporosis [58]. Although in this country there are a number of anti-osteoporotic drugs available, unfortunately these drugs are associated with certain risks and side effects. Additionally, compliance is low and women, in particular, are interested in using alternative/adjunctive therapies including functional foods and their bioactive components.

Our recent findings suggest that dried plum is highly effective in positively modulating bone mass in rat models of osteoporosis [40] as well as improving bone biomarkers over a three-month period in postmenopausal women [19].

More recently, in a preliminary study, dried plum dose-dependently increased steady-state mRNA levels of bone matrix proteins demonstrating the effects of dried plum at the molecular level. The objectives of the present study were to: (1) elucidate the mechanisms by which dried plum polyphenol extract increases bone formation and (2) determine whether dried plum polyphenol extract poses some anti-inflammatory properties. To achieve the first objective, we utilized osteoblast-like cells (MC_3T_3-E_1 cell-line sub-clone 4 from mouse culveri). For examining the anti-inflammatory properties of dried plum, we used lypopolysaccharide (LPS)-stimulated macrophages.

1.2 Research Objectives

1.2.1 Objective 1

In order to find the most effective dose of polyphenol extract in bone mineralization and bone formation, MC_3T_3-E_1 cells were treated with different doses 0, 0.5, 1, 10, 100, and 1000 µg/ml of plum extract. Cells were treated for 15 days and thereafter, alkaline phosphatase, a marker of bone formation, were measured in cell supernatants. Bone mineralization were assessed at fifteen days by staining the cells with alizarin red. Griess assay were performed every three days during a fifteen-day period on the supernatants treated with different concentrations of polyphenol extract. We also attempted measuring nitric oxide and cyclooxigenase (COX-2) in these cells to assess the anti-inflammatory property of polyphenol extract. However, since the number of MC_3T_3-E_1 cells were limited, we predicted that we may not be able to assess these parameters in theses cells. Hence, the same parameters were assed using macrophages as discussed under objective 2. (see Figure 1)

1.2.2 Objective 2

For this objective, RAW 264.7 macrophages were stimulated with LPS. Cell viability were measured using 5 different concentrations of polyphenol extract (0, 1, 10, 100, or 1000 µg/ml) in the presence (1µg/ml) or absence of LPS, known to induce inflammation, at 24 and 48 hours. Furthermore, the dose-dependent effects of dried plum polyphenol extract on pro-inflammatory cytokines such as COX-2 were assessed. (see Figure 1)

1.3 Hypothesis

We hypothesized that polyphenols, the active components in dried plums, enhancing bone formation and reducing the production of pro-inflammatory molecules**.** To test this hypothesis we proposed two specific aims as follows:

1.3.1 Aim 1

To examine the dose-dependent effects of dried plum polyphenols extract in increasing bone formation and mineralization by using osteoblast-like cells (MC_3T_3-E_1).

1.3.2 Aim 2

To evaluate the dose-dependent effects of dried plum polyphenols on pro-inflammatory molecules such as nitric oxide and cyclooxygenase-2 using macrophage cells.

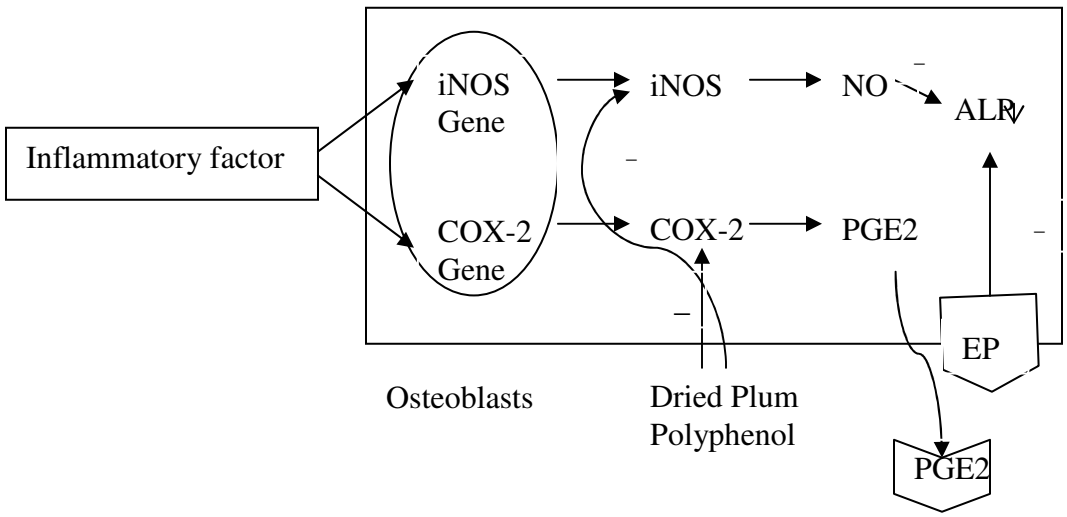


Figure 1. Schematic mechanism of actions of inflammatory factors on osteoporosis and stimulation/inhibition mechanism by which dried plum polyphenols may influence these pathways.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Osteoporosis

By the end of next decade, approximately one in four Americans will be 65 years of age or older and the number of people aged 85 years will double during this period [43], [123]. Trends in population aging have been accompanied nationally by an increased concern for the health-related needs of older people. Age-related bone loss that results in osteoporosis is a significant health problem in the elderly population [60]. The prevalence of age-related bone loss is greater in women than in men [95],[124] and in 25 to 30% of aging women the loss results in major orthopedic problems [22]. In the United States alone, a conservative estimate is that 30 million people have osteoporosis that results in more than 1.2 million fractures per year in people 45 years and older [1]. About 50,000 deaths result from hip fractures annually and the cost to society of osteoporosis and related problems has been estimated at over $18 billion dollars per year [46]. Therefore, osteoporosis is an enormous public health problem with immense socioeconomic significance.

Although osteoporosis is common in elderly women, men also have an increased risk of osteoporosis with aging [71]. Whereas osteoporosis has been studied mostly in women, there is paucity of such studies in men. Age-related bone loss in men has been recognized for many years but its pathogenesis has not been understood. Over the years several hypotheses of the etiology of osteoporosis due to androgen deficiency have been proposed. These hypotheses share in common the association of androgen deficiency with

alterations in the levels or activities of the calcium regulating hormones, but with differing emphasis on the relative contribution of parathyroid hormone (PTH), calcitonin, and 1,25 dihydroxyvitamin D (1,25$(OH)_2$vitamin D) [117], [84]. Currently, none of the hypotheses has been adopted exclusively of the others and the mechanisms by which androgens exert their effects on bone and calcium homeostasis remain unclear. Osteoporosis due to aging may be associated with intestinal malabsorption of calcium [82], [101], the major building block of bone. Although impaired calcium absorption both in males and females is believed to aggravate negative calcium balance and contribute importantly to bone loss in osteoporotic patients [63], other dietary and lifestyle factors aside from calcium, vitamin D and weight-bearing exercise remain unexplored. However, there are a number of other factors that may have profound health benefits on bone including functional foods and their bioactive components which, in part, is the focus of this project. Pant bioactive components have been referred to as phytochemicals. Phytoestrogens, are phytochemicals that are structurally similar to estrogen and may act as either as weak estrogen agonists or antagonist depending on the tissue and the estrogen level [34].

2.1.1 Molecular and cellular mechanism of estrogen on bone

Before the findings of Women's Health Initiative study become publicized [48], estrogen was the most important sex steroid for maintaining bone, as suggested by the beneficial use of estrogen replacement therapy used to treat postmenopausal bone loss [48]. Estrogen acts through both estrogen receptor alpha (ERα) and beta (ERβ). Estrogen receptor α and β share a conserved deoxyribose nucleic acid (DNA) binding domain, a moderately conserved ligand binding domain but a variable N-terminal A/B

domain that contains a transactivation activity (AF-1) that is responsible for interacting with proteins of the core transcriptional machinery [104]. These receptors are nuclear receptors so they are located on the osteoblast and osteoclast nuclear membrane. Estrogen moves into the cell via diffusion and binds to the AF-2 site located in the ligand-binding domain [90], [73]. Estrogen is 245 angstroms, which allows for a perfect fit within the cavity for binding [118]. Hormone recognition is by hydrogen bonds and the non-polar properties of estrogen [105] The steroid is enveloped in a hydrophobic pocket, 450 angstroms, which is sealed by the helix 12 [30]. This closure is essential for the recruitment of co-activators to the AF-2 site and subsequent initiation of the ribose nucleic acid (RNA) polymerase and transcription [30]. The two receptors can bind to the estrogen response elements (ERE) in DNA which they may act as either homodimer or heterodimer α /β to initiate transcription [30]. Or they can bind to the AP-1 site on the DNA via the proteins Fos and Jun [28]. Since ERα and ERβ adopt distinct conformations upon binding, it is hypothesized that this will determine the repertoire of protein cofactors interacting with each ligand-activated, DNA-bound receptor which then participates in the transcription initiated complex [30].

Extensive evidence suggests that estrogen directly modulate osteoblast activity, indirectly regulate osteoclast activity, and regulate a coupling talk between osteoblasts and osteoclasts [78]. However, it remains controversial whether estrogen directly stimulates bone formation or its effects occur secondary to a tight coupling of formation and resorption. The most consistent results demonstrate that estrogen increases the type I collagen formation and the production of transforming growth factor-β (TGF-β), and alters the production of cytokines IL-1, IL-6, and tumor necrosis factor (TNF) [72].

Inconsistent research results may reflect low, variable levels in cells secondary to culture and isolation method plus estrogen demonstrates atypical responses based on the varying artificial ER levels [142]. Overall, osteoclasts have less investigational studies secondary to limited number in bone and difficulty obtaining cells [114]. The most accepted mechanism of estrogen action on bone is the regulation of bone resorptive cytokine synthesis by osteogenic and monocyte/macrophage lineage [114]. However, controversy exist over which is the dominate cytokine regulated by estrogen and which is responsible for the increase in bone resorption in estrogen-deficient conditions [114]. There are potential mechanisms that try to explain the regulation of osteoclasts: estrogen inhibits osteoblastic synthesis of IL-6 which regulates osteoclastic activity and osteoblastic activity [114] and estrogen stimulates monocytes and macrophage synthesis of IL-1 and TNF-α which cause increased resorption. Supportive evidence comes from an investigation that demonstrates that a blockade of IL-1 and TNF-α completely prevents ovariectomized-induced bone loss in rats [114]. These mechanisms are not mutually exclusive, yet all three cytokines may be involved in culminating increased resorption [114]. Overall, estrogens action on bone may control proliferation and differentiation of preosteobasts and osteoclasts; activity of mature osteoblasts and osteoclasts; “coupling” between mature osteobasts and osteoclasts; “coupling” between preosteoblasts and preosteoclasts; production of bone-resorbing cytokines by osteoblasts; osteoclasts, monocytes, and macrophage linage cells [114].

2.1.2 Expression of estrogen receptors in bone

Estrogen receptor α and β are localized in the epiphysis growth plate chondrocytes in humans and rodents; however, the relative abundance is not known [76]. It has been reported the ER-α predominates in trabecular osteoblasts versus cortical osteoblasts; however, lines the osteoclasts and osteocytes with lower levels [138]. The cellular distribution of ER-β is unknown; however, higher levels have been reported in trabecular bone versus cortical bone [137]. In comparison of the two ERs, ERα was the predominant form expressed in the osteoblast precursors (bone marrow stromal cells) and in the trabecular bone [122]. What factors regulate the expression of ERα and β and the changes that occur in different pathological conditions is unknown [141].

2.2 FDA-approved Medications for the Pharmacological Treatment of Osteoporosis

Current approved drugs by the US Food and Drug Administration (FDA) include bisphosphonates (alendronate, ibandronate and risedronate), calcitonin, estrogens, parathyroid hormone and raloxifene which are approved for the prevention and/or treatment of osteoporosis (NOF,2006).

2.2.1. Hormone replacement therapy

Since the use of estrogen replacement therapy or hormone replacement therapy (HRT) is no longer clinically relevant in US or other countries, this subject will not be discussed in details. For instance, Thunell and colleagues (BJOG, 113(1):15-20, 2006) studied the changes in attitudes, knowledge and management strategies concerning hormone replacement therapy (HRT) among 1,323 practicing gynaecologists in Sweden using questionnaire concerning their attitudes, knowledge and management strategies

concerning HRT. They were asked to complete this questionnaire at two different time points (1996 and 2003). They were also asked about their own use of HRT. The response rate was 76% in 2003 when 11% of the gynecologists thought that all women without contraindications should be offered HRT compared with 44% in 1996 and 89% found it difficult to evaluate pros and cons with HRT in a clinical situation (74% in 1996). A great number of gynecologists in 2003 also believed that HRT increased the risk for breast cancer (95% vs 71%). Twenty-five percent of gynecologists in 2003 indicated that risk factors for osteoporosis were the sole indications using HRT (60% in 1996). Current ischaemic heart disease was considered to be an indication for HRT by 7% in 2003 (60% in 1996). In 2003, the use of HRT was reported by 71% of postmenopausal gynaecologists (88% in 1996). In summary, the dindings of this study indicate that Swedish gynaecologists similar those in US are more cautious in their management strategies concerning prescribing HRT in 2003 compared to1996. This change in attitude of both gynecologists and patients is largely influence by the findings of Heart and Estrogen/Progestin Replacement Study [59] and Women's Health Initiative [64] studies.

2.2.2. Bisphosphonates

Bisphosphonates are structural analogues of pyrophosphate. Their metabolic actions inhibition of hydroxyapatite formation, inhibition of bone resorption and alteration of serum phosphorus levels [67]. There are several bisphosphonates; however, the major drugs for osteoporosis are alendronate, etidronate, and risedronate. Bisphosphonates interfere with osteoclast action [119] and have demonstrated a number of effects on the osteoclasts: disrupt the formation of cytoskeletal action ring in polarized,

resorbing osteoclasts [120]; inhibit protein tyrosine phosphatases [107] and induce apoptosis [106].

Etidronate was the first bisphosphonate to be tested in osteoporosis [57]. Later a large randomized controlled trial was published with the bisphosphonate, Alendronate [115]. Alendronate, trade name Fosamax, by Merck Phamaceuticals, was FDA approved in 1996 for the prevention and treatment of osteoporosis. Daily treatments of alendronate have shown to reduce bone loss and increase bone density in the spine and hip of postmenopausal osteoporotic women [65]. The osteoclasts increased bone resorption rate was inhibited and allowed for the usual bone turnover rate to resume without suppressing bone mineralization. Side effects include nausea, constipation, diarrhea, and abdominal pain. The third bisphosphonate used for the treatment and prevention of osteoporosis is actonel, risedronate, which was approved by the FDA in April 2000. This is classified as a bone-resorptive inhibitor.

2.2.3 Raloxifene

Raloxifene is included in a group of compounds that are collectively considered selective estrogen receptor modulators (SERMs). SERMs are nonsteroidal compounds that possess agonist and antagonist properties of estrogen action on the ER [94], [93]. The agonist or antagonist activity of a particular SERM may be related to the class of ER present in the cell or how the conformation of the ligand-ER complex is recognized [80].

Raloxifene is a benzothiophene derivative that was approved by the Food and Drug Administration (FDA) in December 1997 for the treatment of osteoporosis. Raloxifene is relatively a new generation SERM that has demonstrated estrogen-like

effects on the skeleton and cardiovascular system, but antiestrogen effects on the breast and endometrium [2].

A limited number of studies with postmenopausal women showed that a 60 mg dose of raloxifene significantly reduced markers of bone metabolism, alkaline phosphatase osteocalcin and urinary type I collagen C-telopeptide [131], [98]. Beneficial results were also reported with increased bone mass in lumbar spine, total hip, and total body with treatment of raloxifene in postmenopausal women [97]. Not only has raloxifene demonstrated beneficial results with bone metabolism and bone mass but also serum lipoproteins.

Raloxifene has an estrogenic agonist effect on the serum lipoproteins. In a study of postmenopausal women, raloxifene lowered low-density lipoprotein (LDL) to a similar extent as HRT; however, it did not show an increase in HDL as that observed with HRT [132]. This study also showed some beneficial effects of raloxifene over HRT: triglycerides (TG) levels did not increase with raloxifene whereas HRT increased TG and raloxifene lowered serum fibrinogen compared with no effect from HRT.
Breast cancer and uterine cancer where also monitored during particular studies of raloxifene and its efficacy in osteoporosis. One of the larger studies Multiple Outcomes of Raloxifene Evaluation (MORE) included 7,704 postmenopausal women with a meanage of 65 years reported a 74% reduction in risk of breast cancer [35]. Studies of postmenopausal women have not shown to affect the endometrial thickness [38]; however, other evidence has reported an increase in uterine weight and uterine epithelial thickness in ovariectomized rats [49].

Overall, the changes observed with SERMs are often less marked than those observed with HRT. The risk-benefit of the emerging SERMs needs to be better defined and evaluated.

2.2.4 Calcitonin

Calcitonin is a polypeptide hormone made in the C cells in the thyroid [113]. It acts on the osteoclast by inhibiting the proliferation of progenitors as well as the differentiation of committed precursors [134]. Calcitonin is classified as an antiresorptive agent, which was approved by the FDA in 1995 for the treatment of osteoporosis.

2.2.5. Teriparatide

Teriparatide is a trade name for parathyroid hormone (PTH) which is a major calcium-regulating hormone in humans [42]. PTH maintains serum calcium by stimulating bone resorption, regulating renal calcium excretion, and indirectly affecting intestinal calcium absorption[85]. Recent FDA approval of teriparatide, i.e. recombinant PTH, has led to the availability of the first anabolic agent that significantly stimulates new bone formation and reverses bone loss [116]. While the potential for PTH therapy has been received with much enthusiasm, it is not without risk and for many patients may be cost prohibitive [24] teriparatide therapy should be reserved as a last option because of cost and risk associated with its use.

2.3. Other Agents Aside from Calcium and Vitamin D that May Have Bone Protective Effects

2.3.1 Phytoestrogens

Phytochemicals are plant-derived compounds. Among the phytochemicals are phytoestrogens, which is a broad group of nonsteroidal compounds of diverse structure that have been shown to bind to the ER in animals and humans [32]. The major types of phytoestrogens are the lignans and isoflavones [77]. There are three main classes of phytoestrogens: Isoflavones such as genistein and daidzein, both of which are found in soy; lignans such as enterodiol and enterolactone, both of which are found in flaxseed; cCoumestans which is found in bean sprouts and alfalfa. Isoflavones are compounds that are structurally and functionally similar to estradiol [125] based on their displayed binding and stimulating effects on the estrogen receptor [126]. Isoflavones are thought to exert both estrogenic and antiestrogenic effects, depending on the tissue in which they act [4]. Using human cell culture bioassays, isoflavone and estradiol demonstrated equivalent levels of bioactivity with the ERβ [79]. A phenolic structure seems to be a prerequisite for binding to ERs which isoflavones contain [47]. More specifically the isoflavone genistein seems to have more binding affinity for ER-β than ER-α [81].

The proposed similarities between estrogen and isoflavone, biochemical structure and function, fueled the speculation and investigations regarding the potential benefits of soy and isoflavone. The ovariectomized rat model is a common animal model that has indicated that soy protein may have a promising protective effect on the ovariectomized rat bone. In 1998, it was initially reported that genistein displayed a bisphasic effect on bone in two models of ovariectomized rats, young rats and lactating rats, both fed a low-

calcium diet Anderson et al. 1998 [5]. After two weeks of treatment for the young rats and five weeks of treatment for the lactating rats, genistein at the lowest dose of 1.0 mg/d helped prevent ovariectomy-induced, bone changes to an extent similar to the effects of conjugated equine estrogens (5μg/d). In 1996, another study by Arjmandi et al. [9] study supported the therapeutic potential of soy protein on rat bone. This study investigated the effects of soy protein in the same ovariectomized rat model. The femoral bone density of the soy treated group was significantly lower than the estrogen administered and sham groups; however, significantly higher than the ovariectomized casein fed group. The bone density of the fourth lumbar vertebra of the soy group and the estrogen group were equal and significantly higher than the casein and sham group. The results suggested a more protective benefit on the trabecular bone than the cortical bone. In 1998, a follow-up study evaluated whether isoflavones in soy protein are responsible for the bone-protective effects [11], Arjmandi et al., 1998 [12]. The treatment groups included a low isoflavone containing soy protein-based diet and normal isoflavone containing diet. The loss of bone mineral density was prevented by the soy diet with normal isoflavone content but not by the low isoflavone content soy diet. These results suggest that the bone protective substrate in the soy is correlated to the isoflavone content. Two other studies suggested that genistein may be the bone bioactive isoflavone. Ovariectomized rats were fed a diet containing 30 μmol genistein/d for four weeks [27]. The dry femoral mass was 12% higher in the genistein fed group compared to the controls. Another study treated ovariectomized rats with 5 and 25 μg genistein/g body wt injected subcutaneously. Genistein significantly reduced ovariectomized tibia bone mineral loss; however, 1 μg genistein/g body wt did not reduce bone loss.

The potential role isoflavones demonstrate on bone in rats lead to investigations that examined its effect on the human bone. In 1998, a study by Potter et al., reported that after 6 months of treatment, the lumbar spine bone mineral density increased significantly in postmenopausal women who consumed 40 g/day soy protein containing 2.25 mg isoflavones/g protein daily compared with baseline values. However, bone density remained the same in the women who consumed the same amount of soy protein but with only 1.39 mg isoflavones/g protein [112] . A recent human investigation of primenopausal women reported that soy isoflavones attenuated bone loss in the lumbar spine [3]. This 24 week study investigated the effects of isoflavone-rich soy protein (80.4 mg aglycone components/d), isoflavone-poor soy protein (4.4mg aglycone components/d) and whey protein. The aglycone components are the unconjugated parent forms of the isoflavones.

The exact mechanism of isoflavone on bone is unknown; however, there have been some proposed mechanisms. One investigation reported that genistein significantly inhibited dibutyryl cyclic adenosine monophosphate induced osteoclast-like cell formation in the mouse marrow culture [55]. The inhibitory action of genistein may involve the cyclic AMP signaling [54]. Another investigation demonstrated that genistein directly suppresses osteoclasts in vitro [53]. From this investigation, it was suggested that isoflavone may induce apoptosis which is mediated through the intracellular calcium signaling [52]. Not only has isoflavone shown a positive effect on suppressing bone loss, yet it has also demonstrated anabolic potential. Genistein and daidzein both indicated an anabolic effect on bone metabolism in rat tissue culture in vitro [51]. Isoflavones may stimulate bone formation and mineralization [50].

2.3.2 Flaxseed and bone

Among edible plant foods, flaxseed is by far the richest source of lignans, which are reported to have both weak estrogenic and anti-estrogenic activities [36]. Lignans are structurally similar to tamoxifen, which has beneficial effects on bone [139]. Flaxseed is also a rich source of polyunsaturated fatty acid (PUFA), especially alpha-linolenic acid (18:3 n-3) [70]. Alpha-linolenic acid may decrease the rate of bone resorption by inhibiting the biosynthesis of prostaglandins [135]. Lignans present in flaxseed may also possess antioxidant properties. Oxygen-derived free radicals, which are formed by a number of phagocytes including monocytes, macrophages, and neutrophils, have been reported to increase in chronic inflammatory diseases, aging, and osteoporosis. *In vivo* and *in vitro* findings indicate that free radicals generated in the bone environment enhance osteoclast formation and bone resorption. Hence, flaxseed may reduce the rapid rate of bone loss experienced by postmenopausal women, in part, by enhancing antioxidant status. We have previously reported [6] that flaxseed can potentially exert positive effects on bone of postmenopausal women. In a follow-up study, we assigned 60 postmenopausal women not on HRT to receive either 40 g flaxseed or a 40 g wheat-based comparative control supplement for three months. Our results indicates no amelioration of serum and urinary biomarkers of bone metabolism with flaxseed. Whether a longer-term study using bioactive components of flaxseed such as lignans or its oil can exert a positive influence on BMD and BMC remains to be explored.

2.4 Evidence for Beneficial Effects of Dried Plums on Bone

Animal studies and a 3-month clinical trial conducted by Arjmandi and colleagues' have demonstrated that dried plum may positively affects bone density and

bone biomarkers. The animal data showed that dried plum (*Prunus domestica L.)* reverses bone loss in two separate animal models. Finding by Deyhim et al. [39];[20] showed that dried plum *prevented* the ovariectomy-induced reduction in BMD of the femur and lumbar vertebra. Serum IGF-1, which is associated with enhanced bone formation, was also increased by the dried plum treatment. In follow up study [40], rats were ovariectomized and allowed to lose bone before the initiation of treatment. Dried plum as low as 5% (w/w), restored BMD to the level of intact rats. More importantly, dried plums reversed the loss of trabecular architectural properties such as trabecular number and connectivity density, and trabecular separation. This observation is unique to dried plums in comparison with soy or its isoflavones[17], [10], flaxseed [7];[88] and apples[16]. According to Lane et al. [83] once trabecular bone is lost, it would be difficult to restore it.

Our other findings [45];[129] have also shown the effectiveness of dried plum in reversal of bone loss due to skeletal unloading. In these studies, rats were either hind limb unloaded (HLU) or remained ambulatory (AMB) for twenty-one days to induce osteopenia. After confirming bone loss duo to HLU, rats were treated with dried plum or injected with PTH. Analysis of BMD and trabecular bone structure by micro-computed tomography (μCT) revealed that dried plum enhanced bone recovery during re-ambulation following skeletal unloading and had comparable effects to PTH[44]; [128]. In addition to bone density, architectural arrangement is also known to affect bone strength according to McCreadie and Goldstein et al. 2000 [92], [62].These animal findings along with significant increases in serum alkaline phosphatase (ALP) and IGF-1 suggest that dried plum acts by enhancing bone formation**.**

In addition to the animal studies, a 3-month clinical trial by Arjmandi and colleagues [8] indicated that the consumption of 100 g dried plum daily by postmenopausal women significantly increased serum markers of bone formation, total ALP, bone-specific ALP (BSAP), and IGF-1 by 12, 6, and 17%, respectively. The increase in BSAP observed in that study is important because some studies have shown that clinically relevant doses of bone forming agents such as sodium fluoride, growth hormone and PTH take several months to moderately increase serum levels of BSAP [66]. In addition to the increase in BSAP observed in a short-term study by Arjmandi et al. 2002 [15], same study also reported that IGF-1 was elevated in women consuming dried plum. The observations of a 6% increase in BSAP and a 17% increase in serum IGF-1 after three months of consumption of dried plum further support our hypothesis that dried plum will significantly improve bone mineralization as proposed in the present study. From antiresorptive point of view, animal findings reported earlier [130], [20] has observed dried plum's antiresorptive properties in rats as assessed by urinary excretion of tritium-labeled tetracycline. Additionally, the findings of three month clinical study by Arjmandi et al. 2002 [14] showed that urinary helical peptide, a marker of bone resorption, was reduced by 10% although not significantly in the group that received dried plum.

Although the exact osteoprotective component(s) of dried plum is unknown, dried plums are rich in phenolic compounds such as neochlorogenic acid and chlorogenic acid, which act as antioxidants[75], [100]. Antioxidants that scavenge potentially damaging free radicals have been shown to inhibit bone resorption and stimulate bone formation [56], [23,127]. Dried plum has received the highest oxygen radical absorbance capacity

(ORAC) ranking among the most commonly consumed fruits and vegetables [91]. More recently, Kayano et al.[74], have isolated several ortho diphenolic and mono hydroxyl phenolic compounds with ORAC values as high as 4.68 mol from dried plum. Therefore, the beneficial effects of dried plum on bone may be mediated partially through its antioxidant properties.

Dried plum also contains significant amounts of boron than most fruits. Boron has been shown to modulate bone and calcium metabolism [103], and play an important role in preserving BMD [102]. A study by Chapin et al. 1997 [31] also showed that rats that were fed diets containing 20-200 mg boron/100g diet had significantly improved vertebral strength. Nonetheless, the boron levels used in that animal study far exceed the upper limit for humans, which is 20 mg of boron per day. One-hundred grams of dried plum deliver about 2 to 3 mg boron which will not add a substantial amount of boron to one's daily intake. Although these limited studies bring forth evidence that boron may be involved in protecting skeletal health, it is doubtful that the boron alone at the levels present in dried plum would substantially affect bone.

Dried plum is also a good source of other nutrients such as potassium and vitamin K [133], all of which have been reported to influence bone. Tucker and colleagues investigated both cross sectional and longitudinal relationships between potassium and BMD using a Framingham Heart Study database and concluded that potassium contributes to the maintenance of BMD in men and women. Additionally, higher intakes of potassium have been shown to reduce bone resorption, particularly in the face of high protein intake [144].

In terms of vitamins, dried plum contains high amounts of vitamin K (i.e. 59.5 μg phylloquinone/100 g) among commonly consumed foods [41]. Vitamin K influences bone health by improving calcium balance [68] and is a co-factor needed for γ-carboxylation of osteocalcin. γ-Carboxylated osteocalcin promotes normal bone mineralization by regulating the growth of hydroxyapatite crystals[69]. A study by Braam et al. 2003 [29] showed that Vitamin K supplementation at the level of 1000 μg daily for a period of three years retarded loss of lumbar spine BMD. This level is nearly 17 times higher than that present in 100 g dried plum.

The above discussion emphasizes that the bone protective effects of dried plum cannot be merely attributed to any one of its components and this is the reason that at this initial stages of this cell culture study used died plum extract rather than purified compounds from dried plum. It is imperative to initially evaluate the beneficial effects of whole extract rather than its isolated components. In support of this statement, Messina et al. 2001 [96] has indicated that "using a single chemical, such as an individual polyphenolic compound or a combination of a few, may hinder, rather than facilitate our understanding of the role of whole foods or plants in bone health."

CHAPTER III

RESEARCH DESIGN AND METHODS

In this study we used two different types of cell lines, osteoblast-like cells (MC_3T_3-E_1 cells) and raw macrophages. MC_3T_3-E_1 cells originated from mouse calvarias (ATCC, VA, USA) and macrophage cells came from mouse bone marrow RAW-264 (ATCC, VA, USA).

3.1 Experimental Design

3.1.1 Objective 1

To examine the dose-dependent effects of dried plum polyphenols extract in increasing bone formation and mineralization using osteoblast-like cells (MC3T3.E_1) Experimental design to satisfy the first objective is given in Table 1.

Table 1. Experimental design for objective 1 on MC_3T_3-E_1 cells

Treatment Group	Treatment	Concentration of polyphenol
1	Complete α-MEM media	0 µg/ml
2	Complete α-MEM media with dried plum extract	0.5 µg/ml
3	Complete α-MEM media with dried plum extract	1 µg/ml
4	Complete α-MEM media with dried plum extract	10 µg/ml
5	Complete α-MEM media with dried plum extract	100 µg/ml
6	Complete α-MEM media with dried plum extract	1000 µg/ml

Number of samples=4

3.1.2 Objective 2

To evaluate the dose-dependent effects of dried plum polyphenols extract on pro-inflammatory molecules such as nitric oxide and cyclooxygenase-2 using macrophade cells Experimental design to satisfy the second objective is given in Table 2.

Table 2. Experimental design for objective 2 on RAW264.7 cells

Treatment Group	Treatment	Concentration of polyphenol + LPS
1	Complete α-MEM media	0 µg/ml
2	Complete α-MEM media with LPS	0 µg/ml+100 µg/ml
3	Complete α-MEM media with dried plum extract and LPS	1 µg/ml+100 µg/ml
4	Complete α-MEM media with dried plum extract and LPS	10 µg/ml+100 µg/ml
5	Complete α-MEM media with dried plum extract and LPS	100 µg/ml+100 µg/ml
6	Complete α-MEM media with dried plum extract and LPS	1000 µg/ml+100 µg/ml

Number of samples=4

3.2 Cell Culture

MC_3T_3-E_1 osteoblast cell-line from newborn mouse calvaria from ATCC (Manassas, VA) were grown in 75 cm^2 flasks at 37 °C in humidified 5% CO_2 atmosphere in α-MEM (Gibco, NY, USA) supplemented with 10% FBS (v/v) and 1% penicillin (v/v). Subcultures were carried out using a 0.25 % trypsin and 0.03% EDTA solution (Gibco, NY, USA). RAW-264 macrophage cell line from ATCC was another type of cell used in this experiment. This cell line is normally used as a model for bone marrow cells. Since

MC_3T_3-E_1 cell line was not a good model for detecting pro-inflammatory molecules, RAW-264 cells were used to detect proinflamatory molecules such as COX-2 and nitric oxide. This type of cells can grow under 10% CO_2 and at 37°C. The media used for this experiment was D-MEM with 1% penicillin/streptomycin from GIBCO Inc. (NY, USA) and 10% fetal bovine serum from GIBCO Inc. Media was changed every 2 days.

3.3 Ultrasound-assisted aqueous methanol extraction of polyphenolics:

In this method, 80% aqueous methanol and ultrasound are used to extract polyphenolics from freeze-dried powdered plant material according to the methods of. Ultrasound-assisted extraction is rapid and efficient method for the extraction of polyphenolics. Low-frequency, high-energy, high-power ultrasound in the kHz range has the advantage of significantly reducing extraction time and enhancing extraction yield.

Ten grams of ground freeze-dried dried plum was accurately weighed was mixed with 100 ml of 80% aqueous methanol in 500-ml Erlenmeyer flask and sonicate for 20 min at room temperature with continual nitrogen gas purging and periodic shaking. Mixtures were filtered through Whatman number 2 filter paper using a vacuum suction a chilled buchner funnel. The filter cake was rinsed with 50 ml absolute methanol and residue was re-extracted with 100 ml of 80% aqueous methanol by repeating pervious steps. Filtrates were transferred to a 1000-ml round-bottom evaporating flask with 50 ml of 80% aqueous methanol. Methanol were evaporated in rotary evaporator under vacuum at 40°C until the volume of extract was reduced to 10 to 30 ml. The concentrate to a 100-ml volume with deionized distilled water. Nitrogen gas were flushed in to the container to prevent oxidation and they were stored at -4°C until analysis

3.4 Separation of anthocyanin and non-anthocyanin fractions:

Two C18 Sep-Pak cartridges were connected and precondition by sequentially passing 10 ml ethyl acetate, 10 ml absolute methanol, and 10 ml of 0.01 N aqueous HCl through the cartridges. Aqueous polyphenolic extract (sample) were filtered through a 0.45-µm PVDF filter. A known volume filtered extract were loaded onto cartridges.

Cartridges were washed with 6 ml of 0.01 N aqueous HCl to remove sugars, acids, and other water-soluble compounds. Cartridges were dried by allowing a current of nitrogen gas to pass through the connected Sep-Pak cartridges for 10 min. They were rinsed with 40 ml ethyl acetate to elute polyphenolic compounds other than anthocyanins and collect in a 100-ml round-bottom flask. The adsorbed anthocyanins from the cartridges with 6 ml acidic methanol and collected in a separate 50-ml round-bottom flask.

The solvents were removed of the non-anthocyanin fraction and anthocyanin fraction using a rotary evaporator under reduced pressure. Ethyl acetate was evaporated at 20°C and methanol at 40°C or under nitrogen. Each fraction were dissolved in 5 ml deionized distilled water, flushed with nitrogen gas to prevent oxidation, and stored at -4°C until analysis.

3.5 Objective 1

3.5.1 Cell viability

Cells were seeded into a 96 well plates with the density of 3,200 cells per well. After 24 hours incubation, cells were treated with different doses of dried plum polyphenol extract (0.0, 0.5, 1, 10, 100, 1000 µg/ml). Cell viability was assessed at 24 and 48 hours using resazurin assay. Twenty µl of 10% resazurin reagent (Sigma, St

Louis, MO) were added to each well and incubated for 3 hours at 37˚C under 10% CO_2 humidified atmosphere. Then the absorbance was measured at a wavelength of 570 nm and a reference wavelength of 650 nm. The absorbance were reported as nm. Similar experiment was carried out using RAW 264.7 cell line to observe the effect of lipopolysaccharide together with dried plum polyphenol extract on cell viability.

3.5.2 Nodules formation and mineralization

Cells were seeded in 6 well plates at density of 75,000 cells per well. Cells were treated with different doses of dried plum polyphenol extract (0.0, 0.5, 1, 10, 100, 1000 µg/ml) for 15 days, cells the cultures were fixed in ice-cold 70% (v/v) ethanol for 1 hour and stained with alizarin red solution (40 mM, pH 4.2 Sigma Inc St Louis, MO) for 10 min at room temperature. Mineralized nodules were counted by "Meta morph" software (Version 6.2.6) at a 10-fold magnification according to the method of Bielfy and colleagues [26].

3.5.3 Alkaline phosphatase activity

After cells reached 80% confluency, they were treated with 6 different doses of dried plum polyphenol extract (see experimental design in Table 1 and Table 2). Media was changed every 3 days and at the same time supernatants were collected, aliquoted and kept at -20°C freezer. The experiment was continued for 15 days. ALP activity was measured utilizing an automated clinical chemistry analyzer (ACE, ALFA Wassermann ,West Caldwell, NJ). In the reaction, the ALP catalyzed the hydrolysis of the colorless organic phosphate ester substrate, *p*-nitrophenylphosphate, to the yellow colored product

p-nitrophenol and phosphate. ALP was measured directly in the culture media without any chemical or mechanical cell lysing. Absorbance was measured at 340 to 692 nm. The values were reported as unit/liter.

3.5.4 Nitric oxide

After treatment, supernatants from MC_3T_3-E_1 osteoblast-like cells and RAW microphages were collected for the measurement of nitrite, an indicator of nitric oxide (NO) production. The concentration of nitric oxide in the supernatant was measured by Griess assay. Briefly, 50µl aliquots of the cell supernatants were incubated with equal volumes of Griess assay reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-napthylethylenediamine dihydrochloride, NED, in water). The absorbance was measured at wavelength between 520 to 550 nm by a mircoplate reader (ELx808 Biotek, MA). Nitrite concentration was derived from a standard curve generated using sodium nitrite (0.1M in water).

3.6 Objective 2

3.6.2 Protein level of Cox-2 in RAW 264.7 macrophages by western blot on

Cells were seeded in 6 well plates at density of 357,800 cells per well, exposed to 1 µg/ml LPS, then treated with different doses of dried plum polyphenol extract (0, 0.5, 1, 10, 100, 1000 µg/ml) as described under experimental design . When cells reached 80% confluency, they were washed with phosphate buffer saline (PBS) twice. Then 300 µL of lysing buffer consists of: 100mmol/L HEPES, pH 7.9; 100 mmol/L KCL; 100

mmol/L EDTA, 100 mmol/L DTT, protease inhibitor cocktail and 10% IGEPA was added to each plate. Plates were then incubated on ice while shaken on a rocking platform for 10 min at 150 rpm. Thereafter, cells were harvested and centrifuged at 15000×g for 3 minutes at 4 °C. Supernatants of cytosolic fractions were collected and stored at -80 °C.

For measuring protein concentration, 200 µL mixtures of bicinchoinic acid and copper (49:1) were added into 5 µL cytosolic fraction using a 96 well-plate. Plates were then covered and incubated at room temperature for 20 minutes while being gently vibrated using a belly dancer (Stovall Life Science, Greensboro, NS). Absorbance was measured at wavelength of 570 nm.

Equal amounts of protein (20 µg) was brought to volume of 12 µL by adding autoclave water, mixed with 12 µL loading buffer (0.125 mol/L Tris, 4 % sodium dodecyl sulfate (SDS) , 20 % glycerol, 10% 2-mercaptoethanol, 0.003% bromophenol blue pH 6.8) and heated at 95-100 °C for 5 minutes. Protein was separated on 8% SDS-polyacrylamide gel for 2 hrs (100v), and transferred to polyvinylidene diflouride (PVDF) membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus for one hour (15v). Membrane was blocked with Tris-buffered saline (TBS, 8mmol/L Tris HCL, 16mmol/L Tris-base, 150mmol/L NaCl) containing 5% skim milk for one hour. After washing in TBS, blots were incubated overnight with a 1:200 dilution of COX-2 (Santa Cruz Biotechnology, CA) antibody overnight. This was followed by 2-hour incubation with 1:2000 dilutions of goat polyclonal antibody (Santa Cruz Biotechnology, CA) in blocking buffer. The protein bands were developed using an Immuno-Star HRP substrate kit (Bio-Rad laboratories, Hercules, CA) and visualized using VersaDoc Imaging System (Bio-Rad laboratories, Hercules, CA).

3.7 Statistical Analysis

All experiments were repeated four times and values were express as **mean ± SEM** of four measurements. Statistical analysis was preformed using analysis of variance (ANOVA) and least square means (LSM) were calculated using PROC-GLM by SAS (version 8.2, SAS Institute, Cary, NC). Difference among the means was considered significant when $p<0.05$.

CHAPTER IV

RESULTS

4.1 Effect of Prune Polyphenol Extract on Cell Viability

The cell viability results for MC_3T_3-E_1 and RAW264.7 macrophage cells are presented in [33,33]and [108], respectively. The resazurin system measures the metabolic activity of living cells. Solutions of resazurin are dark blue in color. Bioreduction of the dye by viable cells reduces the amount of its oxidized form (blue) and concomitantly increases the amount of its fluorescent intermediate (red), indicating the degree of cytotoxicity caused by the test agent. The plates were read by EL_x 808 ultra microplate reader (BIO-TEK INC, Winooski, VT) and the background was subtracted in order to obtain the actual values.

The results of resazurin assay demonstrated the viability of both types of cells was neither affected by treatment (Table 3) nor by LPS (Table 4, Figure 2)

4.2 Effect of Prune Polyphenol Extract on Mineralized Nodule Formation

Mineralized nodule formation in the presence or absence of 5 different concentration of polyphenol extract was determined on day 15 of MC_3T_3-E_1 cell culture. Dense regions appeared on day 6 of culture which was confirmed as mineralization sites by alizarin red staining. The mineralized nodules became progressively more dense and enlarged by day 15 (Figure 5). In contrast, with the added polyphenol, mineralization was detected with the naked eye on day 15 of culture. The intensity of alizarin red staining on day 15 of culture was clearly greater than those of control cells (Figure 5).

4.3 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was measured to study the effect of dried plum polyphenol extract on the osteoblastic differentiation in MC_3T_3-E_1 cells. Alkaline phosphatase activity was determined for cells cultured with 6 different concentrations of dried plum extract on days 3, 6, 9, 12, and 15 of culture in. ALP activity increased gradually through day 6 (P value<0.0001) of culture and decreased by day 15 (P value<0.0001). Nonetheless, on days 6, 9, 12 and 15 of culture, the overall ALP activity was significantly more in all polyphenol-treated cells than in control cells (Tables 5-6).

4.4 Nitric Oxide

Nitric oxide leads to amplification of inflammation and tissue injury. This experiment was done to examine the anti-inflammatory and antioxidant properties of dried plum polyphenol extract, and the effects of polyphenol in LPS-stimulated (1µg/ml) RAW 264.7 cells on the pro-inflammatory and oxidative stress markers such as NO levels. LPS treatment of RAW 264.7 macrophages significantly (P value<0.0001) elevated NO generation by 16 times more than the LPS-untreated cells (Table 8). Dried plum polyphenol extract was found to suppress NO production in concentration-dependent manner (Figure 5). As shown in Table 8 dried plum polyphenol extract dose-dependently inhibited nitrite accumulation in LPS- activated RAW 264.7 cells. After 24 hours of stimulation, LPS-treated cells increase NO production. Interestingly this LPS-activated nitrite production was significantly (P<0.05) reduced by incubation with various concentrations of polyphenol extract (Table 8).

4.5 Inhibition of COX-2 Protein Expression

We further investigated the effect of dried plum polyphenol extract on COX-2 protein expression in RAW 264.7 cells using Western blotting. Our results showed that dried plum polyphenol extract significantly (P, value<0.0001) reduced the protein level of COX-2 (Figure 7).

CHAPTER V

DISCUSSION

In this study, we have demonstrated that mineralized matrix formation is increased by dried plum polyphenol extract treatment in MC_3T_3-E_1 cell line. At the cellular and molecular levels, polyphenol extract have been shown to inhibit osteoblast apoptosis [61], impede cell cycle progression [111], increase nodule formation [109], and upregulated the expression of osteoblastic genes such as ALP [109]. Moreover, nitric oxide can activate COX pathway in endothelial, pancreas islet, in vitro as well as in vivo as previously confirmed by several groups [121, 136]. Other researchers [143, 33] have shown that nitric oxide inhibits ALP activity via PGE2 and that PGE2, in turn, negatively modulates NO production. These results indicate that iNOS induced by cytokines mediates the production of NO. In the present study, we have demonstrated that inflammatory factor such as LPS induces the nitric oxide production and activates the COX pathway. Based on our result, the measurement of metabolic activity of living cells shows that polyphenol extract doesn't have any toxic effect on either MC_3T_3-E_1 or RAW264.7 macrophage cells as viability of these cells were not affected by the doses used in this study.

While polyphenol extract had no effect on cell viability, it dose-dependently increased nodule formation. These results indicate that dried plum polyphenol extract enhance the activity of osteoblasts, especially the formation of hydroxyapatite crystals (Figure 5).

The above findings were further supported by our observations that the activity of ALP on days 6, 9, 12, and 15 was higher in the presence of polyphenol extract in

comparison with control cells. ALP, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in the calcification of bone [89]. Not only does the enzyme hydrolyze substances, it inhibits calcification, such as pyrophosphate and ATP, but it is also vital for producing the increased phosphate concentration required for hydroxyapatite crystallization. These findings are in agreement with those of Arjmandi et al. [21] and Deyhim et al. [40]. The study by Arjmandi and colleagues [21] suggest that dried plum diet was effective in preventing bone loss due to ovariectomy as indicated by higher bone densities, mineral contents, percent trabecular bone area, and tendency to reduce marrow space in comparison with OVX control rats. They [21], [40], concluded that dried plum elevated the rate of bone formation as suggested by a dose-dependent rise in serum IGF-I levels. Higher serum IGF-I concentrations are considered to be reflective of the elevated rate of bone formation.

Deyhim et al. [39], studied the bone reversal properties of dried plum using osteopenic ovariectomized rat model. In that study [40], dried plum, as low as 5%, was effective in restoring femoral and tibial bone density. Dried plum also improved lumbar bone density. The investigators similarly concluded that the improvement in bone density and biomechanical properties of long bones due to dried plum, in part, may be due to enhanced rate of bone formation.

There is also evidence that polyphenolic compounds such as flavonoids [99], have anti-inflammatory effects that may exert beneficial effects on bone by suppressing the production of pro-inflammatory molecules. In the present experiment, dried plum polyphenols dose-dependently inhibited the production of nitric oxide in osteoblast-like

cells and was also able to reduce the protein level of COX-2 in LPS-stimulated macrophages. Therefore, our observations indicate that dried plum polyphenol extract has the capability to effectively suppress the production of pro-inflammatory molecules and thereby enhance bone formation. Dried plum polyphenols may act as powerful inhibitor of COX-2 activity and much of this effect may be through the inhibition of cytokines that mediate adhesion of circulating leukocytes to sites of injury. For example, Lin and colleagues [86,87] reported that flavonoids, a class of polyphenols, are powerful inhibitors of IL-1β, IL-6, TNF-α, and COX-2. Dried plum polyphenols may thus inhibit the inflammatory responses through these modes of action, suggesting that this class of molecules may be effective in conditions such as osteoporosis. However, much of this discussion is speculative and further research is needed to confirm the findings of this cell culture study.

As to what components of dried plum polyphenol extract exert these bones protective effects remain to be answered. Dried plum polyphenol extract similar to whole fruit contain numerous compounds that make it difficult to discriminate the effect of one compound versus another. Dried plum polyphenol extract, in addition to being a good source of nutrients such as certain vitamins and minerals that can influence bone health, are a rich source of phenolic compounds that act as antioxidants. Hence, dried plum polyphenol-rich extract may exert positive effects on bone, in part, through their antioxidative capabilities. Dried plum, as a whole fruit, has been ranked highest in antioxidant values [110] among the commonly eaten fruits and vegetables as assessed by ORAC, an in vitro analysis that measures the total antioxidant capacity of foods and other chemical substances [37].

Furthermore, the polyphenols in dried plum may protect bones similar to other phenolic compounds with estrogen-like activities such as isoflavones [140]. However, this is speculative and as to what extent polyphenols in dried plum have phytoestrogenic properties need to be investigated.

Table 3. Effect of different doses of polyphenol extract on cell viability of MC3T3-E1 at 24 hours and 48 hours as assessed by resazurin assay

Concentration µg/ml	Cell Viability at 24 Hours	Cell Viability at 48 Hours
control	0.697±0.037005	0.79±0.091329
0.5	0.775±0.078271	0.606±0.02669
1	0.726±0.077272	0.605±0.059408
10	0.758±0.078823	0.607±0.071253
100	0.732±0.072542	0.597±0.037873
1000	0.671±0.035595	0.575±0.040673

Values are mean ± SE, *n* = 4. There were no significant differences among the treatments.

Table 4. Effect of different doses of LPS on all viability of RAW 264.7 at 24 hours as assessed by resazurin assay

Concentration µg/ml	Cell Viability at 24 Hours
0 (control)	0.361±0.02375
0	0.360±0.01569
1	0.361±0.02750
10	0.394±0.04666
100	0.392±0.02353
1000	0.392±0.07851

Values are mean ± SE, *n* = 4. There were no significant differences among the treatments.

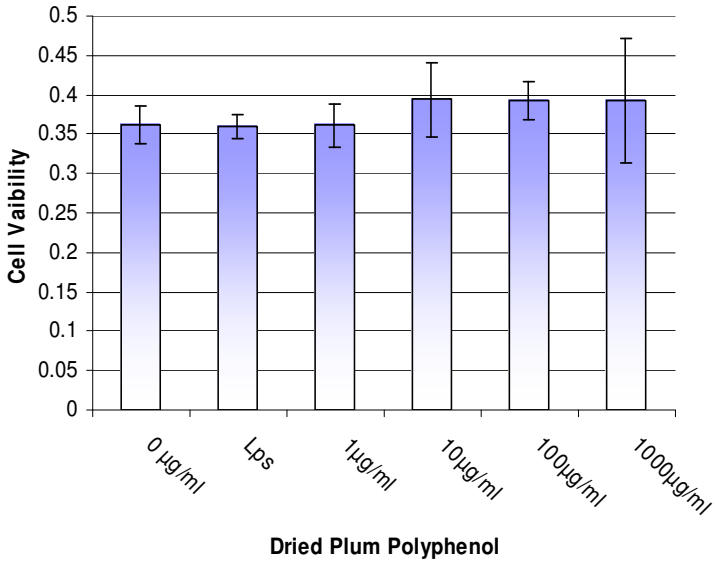


Figure 2. Cell viability of RAW 264.7 effect of different doses of dried plum polyphenol extract at 24 hours as assessed by resazurin assay. There were no significant differences among the treatments.

Table 5. 15 days treatment of effect of different doses of polyphenol extract on alkaline phosphatase activity of MC3T3-E1 cell.

Polyphenol extract Concentration µg/ml	Alkaline phosphatase activity, U/L
0 (control)	1.1655±0.1603[b]
0.5	1.0245±0.1603[b]
1	2.014±0.1603[a]
10	1.098±0.1603[ab]
100	1.9025±0.1603[a]
1000	1.8415±0.1603[a]

Values are Mean ± SE, $n = 4$. Mean values that do not share the same letters are significantly different from each other ($P < 0.05$)

Table 6. ALP activity Unit/L/Day

Day3	Day6	Day9	Day12	Day15
c 0.404±0.1463	b 1.256±0.1463	b 1.452±0.1463	b 1.842±0.1463	a 2.586±0.1463

Values are Mean ± SE, $n = 4$. Mean values that do not share the same letters are significantly different from each other ($P < 0.05$)

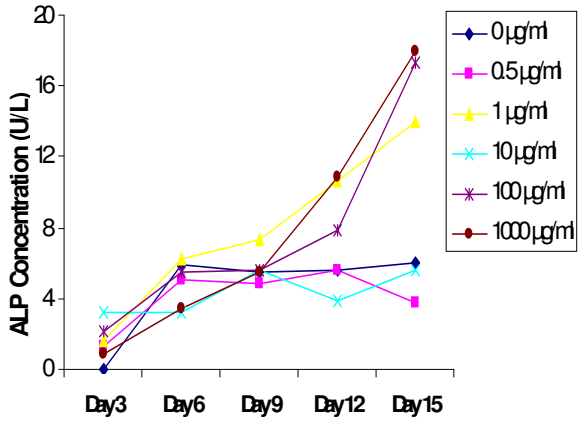


Figure 3. Effect of dried plum polyphenol extract on alkaline phosphatase activity at different time points of MC_3T_3-E_1 cells. Data shown are mean ± S.E.M. ($P < 0.05$).

Table 7. Represents number of nodule formation on day 15th of cell culture. Cells were treated with 6 different concentrations of dried plum polyphenol extract.

Concentration	Number of nodule/well
control	429.9209±12658[e]
0.5µg/ml	266.5793±20009.5[e]
1µg/ml	1755.039±43431[d]
10µg/ml	1686.45±86821.5[c]
100µg/ml	13355.83±110026[b]
1000µg/ml	1576.848±156516[a]

Values are mean ± SE, $n = 4$. Mean values that do not share the same letters are significantly different ($P < 0.05$).

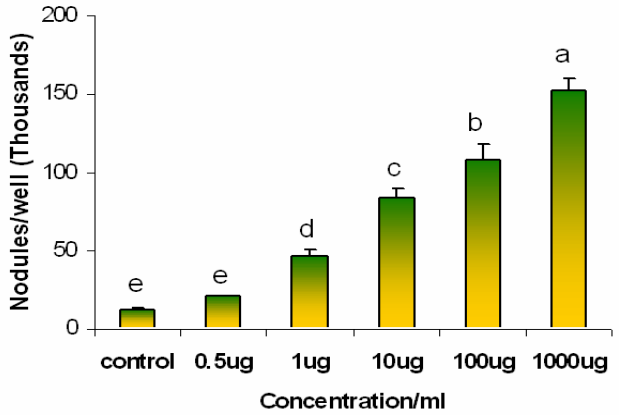


Figure 4. Effect of dried plum polyphenol extract on nodule formation of MC_3T_3-E_1 cells at day 15. Data shown are mean ± S.E.M. ($P < 0.05$).

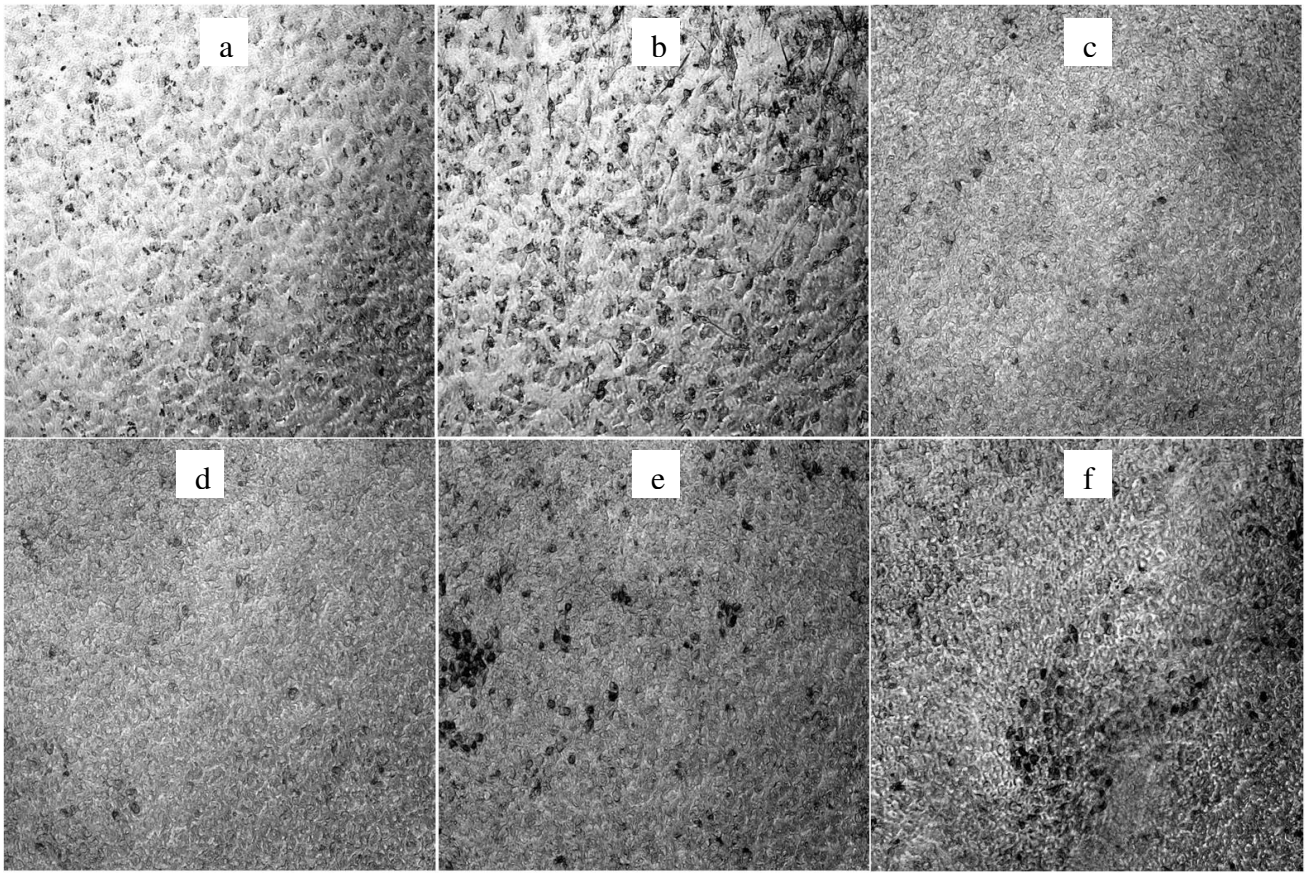


Figure 5. (a) Specific histochemical staining for ALP. Detection of mineralized nodules by alizarin Red S staining in mouse osteoblast cultures grown for 15 days in normal growth medium supplemented with 10% FBS. The images show the differences between the cells cultured in αMEM supplemented with 10% FBS (a) cells were cultured in αMEM supplemented (b) 0.5 μg/ml (c) 1 μg/ml (d) 10 μg/ml. (e) 100 μg/ml, and (f) 1000 μg/ml dried plum polyphenol extract original magnification 10×.

Table 8. Effect of dried plum polyphenol extract and LPS on NO production of RAW 246.7 cells.

LPS Con(1µg/ml) and Con of polyphenol (µg/ml)	**Nitric Oxide (100**ng/ml LPS)
0	0.09±0.02[c]
0	1.44±0.06[a]
1	1.43±0.14[a]
10	1.36±0.15[ab]
100	1.34 ±0.02[ab]
1000	1.20±0.17[b]

Values are mean ± SE, $n = 4$. Means that do not share the same letters are significantly different at $P < 0.05$.

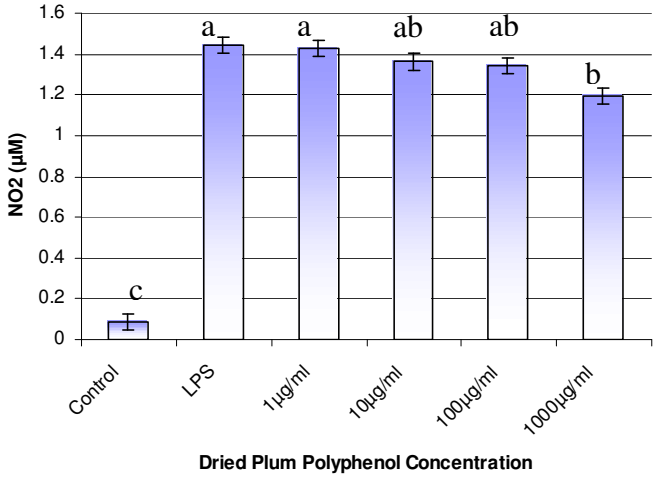
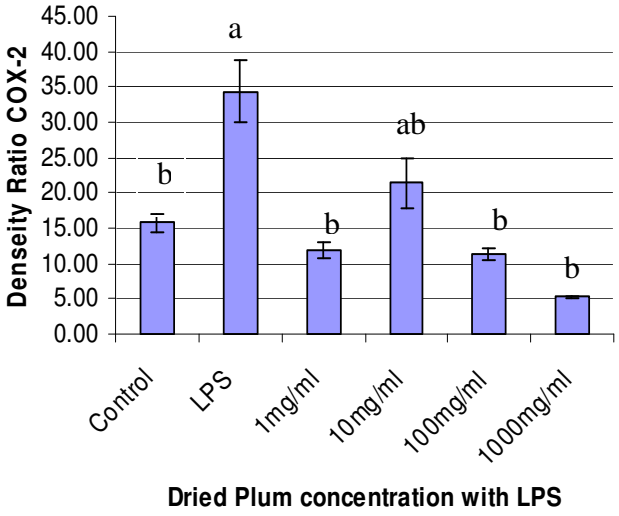


Figure 6. Effect of LPS and different doses of dried plum extract on NO production in RAW 264.7 macrophage.

(a)



(b)

Figure 7. Effect of LPS and various doses of died plum polyphenol extract of protein level of COX-2 as assessed by Western blot.
(a) Represent active bands from the membrane and
(b) dencity of the COX-2 band.

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VITA

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Findings and Conclusions: showed that polyphenol extract decreased the production of pro-inflammatory molecules such as COX-2 and nitric oxide in macrophages. Overall, the finding of the present study suggests that dried plum polyphenol extract enhances bone formation and suppresses the production of inflammatory molecules known to be harmful to bone.

ADVISER'S APPROVAL: Dr. Bahram H. Arjmandi

ABSTRACT

Evidence for Bone Forming ability of Dried Plum Polyphenol Extract

Our earlier findings indicate that dried plums are highly effective in preventing and reversing ovarian hormone deficiency-associated bone loss in a rat model of osteoporosis. Moreover, our short-term clinical trial showed that dried plum supplementation significantly increased indices of bone formation in postmenopausal women. The bone protective effect of dried plum, in part, may be due to its polyphenol content. The intent of the present study was to investigate the bone formation mechanisms of action of polyphenol extracted from dried plum using a cell culture system. We hypothesized that polyphenol extract from dried plum increases bone mineralization in MC3T3-E1 cell line. Also we hypothesized that polyphenol extract decrease pro-inflammatory molecules in macrophages. MC3T3-E1 cells were treated with various doses of polyphenol extract (0, 0.5, 1, 10, 100, 1000 µg/ml). Media was collected every three days and analyzed for alkaline phosphatase activity. After 14 days, the formation of mineralized nodules was characterized by Alizarin red staining. Our findings indicate that dried plum polyphenol dose-dependently increases mineralization without affecting cell viability as shown by resazurin assay. Alkaline phosphatase activity was significantly increased at days six and nine. Among the doses tested, 100 µg/ml of polyphenol extract caused a significant elevation of alkaline phosphatase activity. For the second objective macrophages were stimulated with 100 ng/ml LPS and then they were treated with (1, 10, 100, and 1000 µg/ml) polyphenol extract. Result showed that polyphenol extract decreased the production of pro-inflammatory molecules such as COX-2 and nitric oxide in macrophages. Overall, the finding of the present study suggests that dried plum polyphenol extract enhances bone formation and suppresses the production of inflammatory molecules known to be harmful to bone.